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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 12 (9): 683-689, 2009
ISSN 1028-8880

Bacterial Diversity of Marine Seeps in the Southeastern Gulf of Mexico

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Abstract: The diversity of bacterial communities of shallow (≤ 100 m depth) oil seep marine sediments from the Southern Gulf of Mexico was evaluated. The geochemical properties of seep sediments were characterized as well as their microbial diversity in oil seep and control sediments. Bacteria were identified through molecular tools as belonging to the genera *Marinobacter, Idiomarina, Marinobacterium, Frauteria* and an unknown bacterium. Bacteria might be important components of microbial communities in Total Petroleum Hydrocarbon (TPH)-containing environments, displaying either facultative metabolism or able to grow only in petroleum-containing media. The identification of bacteria in shallow oil seep sediments could be used as indicators of marine hydrocarbons in Southeastern Gulf of Mexico.

Key words: Oil seeps, bacterial community, TPH-containing environments, marine sediments

INTRODUCTION

In Mexico, the largest industrial petroleum facility is located in the state of Campeche, supplying 82% of Mexican oil and 35% of natural gas (García-Cuéllar *et al*., 2004); within the area, the Cantarell oil field harbors several shallow (≥100 m deep) oil seeps with a high hydrocarbon emission potential (Wilson *et al*., 1974). Oil seepage at Cantarell occurs primarily in shallow water areas where faults and salt diapers penetrate overlying sediments, creating migration pathways from source rocks to the sea floor (Pellón de Miranda *et al*., 2004). A well-known oil seep area is located close to the Akal oil rig complex, 70 km offshore North of the island (Isladel Carmen) (Pellón de Miranda *et al*., 2004; Soto *et al*., 2004). Diversity, an important component of any biological community, is strongly linked to species richness, functional diversity and resilience (Von Canstein *et al*., 2002; Tilman and Lehman, 2002). Resilience, the capacity of an ecological system to recover equilibrium after a perturbation (Folke *et al*., 2002), seems to play an important role in the Campeche Bank, Southern Gulf of Mexico, where important fisheries and protected ecosystems surround the region. This was evident in 1979, when a blowout from the oil well Ixtoc-I released to the area nearly 3,100,000 barrels of crude oil. Despite of this disaster; after a year, no effects were seen in the nekton community, the zooplankton biomass and phytoplankton species, as well as in the ratio of hydrocarbon-degrading bacteria to heterotrophic bacteria (Lizárraga-Partida *et al*., 1982).

Archaea and some Eubacteria seem to dominate the microbial communities in deep (>500 m) seep sediments from the Northern Gulf of Mexico (Lanoil *et al*., 2001; MacDonald *et al*., 2004; Mills *et al*., 2005). Recently, the study of deep subseafloor aerobic microorganisms was approached in methane-rich sediments, identifying bacteria with high enzymatic diversity (Kobayashi *et al*., 2008). However, very little is known about the bacterial diversity in sediments of shallow seeps in the Southern side. In the present study we approached the characterization of the bacterial community present in the Cantarell (Akal) seep and evaluated its diversity using microbiological and molecular tools. The identification of these bacteria as indicators of TPHs is proposed in the present manuscript.

MATERIALS AND METHODS

Location of seeps and control areas: A federal restricted oil rig area where natural Total Petroleum Hydrocarbons (TPH) emanations occur. was selected for the analysis. Two sampling sites were selected, an oil seep location (named here S) close to the Akal H oil rig, at 92° 20'and a control location outside of oil seepage 93° 40' (Fig. 1) . Both sites are located at 42 m depth within the Cantarell oil field in the Southern Gulf of Mexico and were

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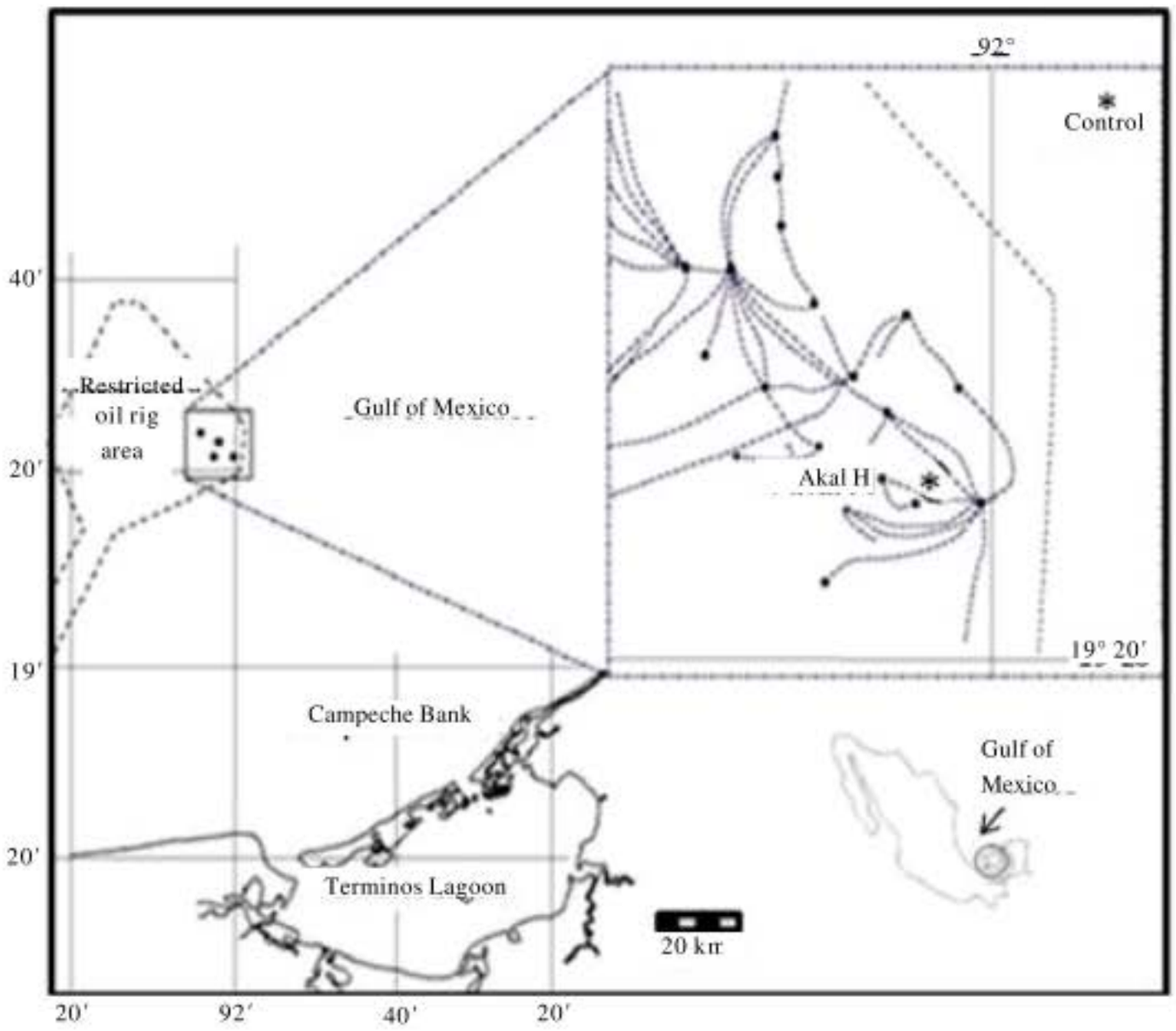

Fig. 1: Area of study within the Cantarell oil field, Southern Gulf of Mexico. Solid circles represent oil rings; dotted lines are oil or gas pipelines

sampled in the summer of 2006. The sediment samples were collected with a mechanical driller, producing cylindrical cores (ca. 25 m long), which were then transported to sterile conditions in the lab for further analysis.

Sediment sampling, geochemical parameters and TPH analysis: Sediment samples were collected with a mechanical driller, producing cylindrical cores (ca. 25 m long). Each sediment core was sectioned at different depths on board and sediment core sections were transported at 4°C to the laboratory for further processing as recommended by Exon *et al*. (2001). Samples from the center of each core section were collected under sterile conditions and used to quantify TPH, using the US EPA method 418.1. Samples were also characterized in terms of their bacterial content, bacterial *in vitro* propagation and total DNA extraction.

Estimation of the source generative potential and the type of hydrocarbon present in the seep was performed in 100 mg of selected sediment samples. For this purpose, Rock-Eval pyrolysis parameters (S1, S2 and S3) and other geochemical parameters such as Total Organic Carbon (TOC) and Hydrogen (HI) were evaluated according to Peters and Cassa (1994).

Quantification and isolation of cultured bacteria: Under sterile conditions, 1 g of sediment from each core was diluted ten fold with sterile, artificial seawater and adjusted to 36 parts per thousand (ppt) of salinity (Lyman and Fleming, 1940). Decimal dilutions were plated in triplicate plates on both peptone agar 2216E and Mineral Agar (MA) supplemented with heavy (API>30) Maya oil from the Campeche Bank as the only carbon and energy source. The MA composition was, for 1 L: 360 mL artificial seawater; 640 mL distilled water; 1 mL $FeCl_3$ 0.12% (w/v); 1.5% bactoagar (Difco); pH 7.5. An emulsion was formed by agitating the MA and the TPH and immediately poured in plates, which were then inoculated and incubated at 30°C for 48 h. Colony forming units (cfu) were observed after 48 h.

***In vitro* bacterial propagation:** Bacterial colonies with different morphology were selected and then aerobically grown in liquid media, supplemented with 0.5% (v/v) Maya oil or peptone broth. Flasks were then incubated at 30°C, at 180 rpm in the dark for 10 days.

DNA extraction from sediments and cultured bacteria: Metagenomic DNA from 0.5 g of the sediment cores and DNA from the *in vitro* propagated bacteria was extracted using the Ultra Clean Soil DNA Isolation Kit (Mo Bio Laboratories, Solana Beach, USA) following the manufacturer's instructions. Purified DNA was employed as template for amplification of the 16S-23S ribosomal spacer plus a stretch of the 16S rDNA (500 bp) as described by Acinas *et al*. (1999) and Martin (2002). Primers employed were B1055-16S (5'-AATGGCTGTCGT CAGCTCGT-3') and 23SOR (5'-TGCCAAGGCATCCA CCGT-3'), which anneal to positions 1055-1074 of the *E. coli* 16S rDNA and 21-38 of the 23S rDNA genes, respectively (Amann *et al*., 1995; Gürtler and Stanisich, 1996). PCR reactions were performed in a Biometra T-gradient thermal cycler (Göttingen, Germany), with the Eppendorf Master Mix Kit (Eppendorf, Germany). Aliquots of each PCR product were resolved in 4-20% gradient polyacrylamide gels (Ready Precast Gels, BioRad, Hercules USA) and stained with the Silver Stain plus Kit (BioRad).

Analysis of PCR-RISA banding patterns: The expected size of amplified region ranged from 0.6 to 1.6 kb. Each amplified band of the rDNA Intergenic Spacer Region Analysis (RISA) was assumed to represent a different genotype. Accordingly, Shannon-Weaver and Simpson diversity indices were calculated using risotypes as Operative Taxonomic Units (OTUs), as implemented by Krebs (1999). Dendrograms were calculated with the program NT SYS using the band pattern obtained by PCR RISA. Cluster analysis was performed with simple matching, with the linking method SAHN (Fingerprinting II, Biorad).

Construction of rDNA libraries: Amplified DNA fragments were cloned into the pDrive vector kit (Qiagen, Santa Clarita, CA) and transformed into *Escherichia coli* One Shot TOP10 competent cells (Invitrogen, Carlsbad, CA). Recombinant clones were then cultured, following standard procedures (Sambrook and Russell, 2001). Plasmid DNA purification was carried out using the FastPlasmid Mini kit (Eppendorf).

Sequence analysis and taxonomic determination: Purified, recombinant plasmids were sequenced with the B1055 primer by using the Big Dye Terminator kit version 3.1 (Perkin-Elmer) and the 3100 ABI PRISM sequencer (Perkin-Elmer) in Langebio CINVESTAV IPN, México. DNA sequences were analyzed with the Bellerophon chimera detection program (http://foo.maths.uq.edu.au/~huber/bellerophon.pl). Multiple alignments using Crustal X were calculated with the DNA sequences and 16S rRNA sequences deposited in the GenBank. All sequences were adjusted to a similar size (approx. 400 bp), sharing the same rDNA 16S region. GenBank nucleotide sequence accession numbers for the sequenced clones are EF143342 to EF143350. Bacterial identification was established when DNA homology with the extant databases was assessed with confidence values bigger than E = 0.9 (Montalvo-Hernández *et al*., 2008).

RESULTS

Total Petroleum Hydrocarbons (TPH) and the hydrocarbon generative seep potential: The area for sampling was selected for the presence of bubbling and oil slicks observed in the sea surface of the seepage area. A detailed analysis on its TPH content, based on Rock-Eval pyrolysis parameters, allowed the estimation of TOC (Total Organic Carbon) 1.6-1.9 and HI (Hydrogen Index) 169-414, which is consider to be high from values 200 (Table 1) indicating the presence of marine organic matter. This location is considered a natural spring of oil and gas (Broadhead *et al*., 1998). TOC (1.9 wt.%), HI (414 mg HC g^{-1} TOC) and Rock-Eval parameter values (RE1 = 1.2 mg HC g^{-1}; RE2 = 7.9 mg HC g^{-1}; RE3 = 3.2 CO_2 g^{-1}) were obtained in the sample S4, located to only 5.8 m (Table 2). Interestingly, in this sample S4, six genotypes were identified as able to grow in TPH-containing media (Table 4). In contrast, control sediments, with no macroscopic evidence of hydrocarbon deposition, a limited TPH, as well as a limited TOC content or hydrogen index was observed (Table 3);bearing values similar to those present in background values in marine sediments (Blumer and Sass, 1972). For applied purposes, the characterization of such seeplocation suggests the presence of potential,

Table 1: Geochemical characterization of total petroleum hydrocarbons extracted from seeps and control sediments

Sediment location	Seep	Control
Depth (m)	2.1-19.8	2.1-19.7
TPH*	194±96	0.2±0.04
TOC*	1.6-1.9	0-1.3
HI*	169-414	36-150
Rock-Eval pyrolysis		
RE1	0.2-1.2	0.0-0.1
RE2	2.7-7.9	1-1.3
RE3	2.8-3.2	0.8-3.4
Source generative potential	Good	Poor-fair
Hydrocarbon generated	Gas and oil	Gas

*Total petroleum hydrocarbons (g kg^{-1}), *Total organic carbon (%w), *Hydrogen index, quantity of pyrolyzable HC from S2, relative to the TOC sample (mg HC g^{-1} TOC), Rock-Eval pyrolysis para meters: S1, Milligrams of hydrocarbons thermally distilled from 1 g of the sediment; S2, Milligrams of hydrocarbons generated by pyrolytic degradation of the kerogen in 1 g of sediment; S3, Milligram of carbon dioxide generated in 1 g of sediment during temperature programming up to 390°C

superficial area of TPH emanations, with a good source generative potential and adequate site for gas and oil production (Table 1).

Seven subcores were sampled and TPH were determined as described in material and methods (Table 2). The subcores of the seep samples, named S1 to S7 were obtained from 2.1 to 19.8 m in the seep, while five core control samples C1 to C7, from 2.1 to 19.7 m outside the seep were also collected. TPH were located in discrete strata in the analyzed column, where the highest TPH concentration was estimated in 296 g kg^{-1}. In contrast, TPHs accumulated in three orders of magnitude lower in controls, estimated in only 0.2 units (Table 2).

***In vitro* propagation of culturable, heterotrophic and TPH-degrading bacteria from seeps:** Estimation of colony forming units (cfu) as a measure of culturable bacterial was performed from both seeps and control samples (Table 2). Both heterotrophic and hydrocarbonoclastic populations, obtained from strata with different deepness, were grown in both solid media and obtained colonies were calculated as cfu×10^8 in 1 g of dry sediment as indicated in material and methods (Table 2). As suggested by the geochemical characterization, a high variation was observed in heterotrophic growth, ranging from no growth at 19.8 m to 52×10^8 cfu g^{-1} in the strata 4 (5.8 m) when incubated in rich media. In contrast, hydrocarbon-degrading bacteria were quantified from low variation to no growth, in two to three orders of magnitude higher than earlier reports for sediments of the same region (Lizárraga-Partida *et al*., 1982). It is important to mention the obtained calculations account for cultivable bacteria and no experiments were approached to calculate the total population present in such particular habitats. In order to measure bacterial diversity, both bacterial populations were statistically compared and the one-way ANOVA showed significant differences between C and S locations ($p<0.5$). Student t-test also confirmed a statistically significant difference at $p<0.5$. Concerning the distribution in sediment strata, bacteria were heterogeneously distributed along the columns. In C and S locations, the distribution of both heterotrophic and TPH-degrading bacteria from 2.1 to 11.3 m was significantly influenced by depth ($p<0.5$); except for heterotrophic, in the first layer located at 2.1 m (Table 2).

Diversity and bacterial community structure in oil seep sediments: Shannon (H) and Simpson (D) Diversity indices were calculated from both sites. A global comparison showed that the bacterial diversity was slightly higher in the seep sediments (H = 2.52, D = 0.82), than outside, control areas (H = 2.30; D = 0.81) (Table 2). Based on the RISA profiles, the composition of the bacterial communities varied also between seep and control locations, as well as among strata along the seep sediment column (Table 2). In sample 7 where the TPH

Table 2: Bacterial diversity in seep and control sites sediments

Sample	Depth (m)	TPH* (g kg^{-1})	Bacteria heterotrophic	TPH-degrading #(cfu 10^8 g^{-1})	Indices	
					Shannon	Simpson
S1	2.1	50	49±7	4±0.5	1.6	0.7
S2	4.3	0.9	14±1	12±1	2.0	0.7
S3	5.2	0.8	4±1	5±0.5	2.0	0.7
S4	5.8	1	60±15	2.5±0	2.0	0.7
S5	11.3	166	0.3±0	0.3±0	1.0	0.5
S6	18.3	262	0	0	nd	nd
S7	19.8	296	0	0	1.0	0.5
C1	2.1	0.2	44±15	0.5±0.1	1.6	0.7
C2	3.1	0.2	6±0.5	4±1.5	2.3	0.8
C3	4.9	nd	48±11	2±0	0.0	0.0
C4	5.5	0.2	13±2	2±0	2.3	0.8
C5	10.1	nd	6±2	2±0	0.0	0.0
C6	18.1	0.2	0	0	1.6	0.7
C7	19.7	0.2	0	0	nd	nd

*TPH: Total petroleum hydrocarbons, #Colony forming units ×10^8 g^{-1} of dry sediment, nd: Not determined

Table 3: Summary of amplified rDNA from seep and control strata

Sample	Depth (m)	TPH (g kg^{-1})	ITS DNA (kb)
S1	2.1	50	0.8, 0.9, 1.2
S2	4.3	0.9	0.9, 0.95, 1.5, 1.6
S3	5.2	0.8	0.8, 0.95, 1, 1.2
S4	5.8	1	0.65, 0.7, 0.9, 1.6
S5	11.3	166	1, 1.45
S6	18.3	262	nd
S7	19.8	296	1.55, 1.6
C1	2.1	0.2	0.95, 1.5, 1.6
C2	3.1	0.2	0.8, 0.9, 0.95, 1.5, 1.6
C3	4.9	nd	0.95
C4	5.5	0.2	0.75, 0.8, 0.85, 0.9, 0.95
C5	10.1	nd	1.2
C6	18.1	0.2	0.9, 0.95, 1.35
C7	19.7	0.2	nd

nd: Not determined

Table 4: List of identified *in vitro*-propagated genotypes

Accession No.	Source	DNA (bp)	Homology (%)	Family	Genus
EF143342	Seep S4	409	99	Alteromonadaceae	*Marinobacter* AY869156
EF143343	Seep S4	409	99	Alteromonadaceae	*Idiomarina* EU624436
EF143344	Seep S4	407	99	Alteromonadaceae	*Idiomarina* EU624436
EF143345	Seep S4	409	--	Unknown	Unclassified
EF143346	Seep S4	409	99	Alteromonadaceae	*Marinobacterium*
EF143348	Seep S4	410	99	Xanthomonadaceae	*Frateuria* AB264175
EF143347	Control C4	409	99	Vibrionaceae	*Vibrio* sp.
EF143349	Control C4	410	98	Xanthomonadaceae	*Fulvimonas* AJ311653

accumulated to high levels (296 g kg^{-1}) no bacteria could be propagated *in vitro*. However, direct rDNA amplification from this sample yielded two bands, suggesting the presence of habitat-restricted bacteria, uncultivable in the tested media. This concentration definitely prevented other TPH-degrading organisms from growing; in contrast, lower TPH concentrations present along the column showed detectable heterotrophic and TPH-degrading bacteria (Table 3). Bacterial community structure was then estimated by calculating a conglomerate tree as described in material and methods. Figure 2 shows the structure of bacterial populations calculated from RISA profiles within the seep area (A) and control, no TPH-containing strata. Interestingly, a diverse location of seep-privative TPH-degrading bacteria within the tree was observed in S3, S4, S5, S7 (Fig. 2), as five risotypes of 0.65, 0.7, 1, 1.45 and 1.55 kb were only present within the seep location, regardless of their TPH-content. Both communities are diverse, but, on a speculative note, differences in metabolic capacities could explain their ability to use TPH as a source of carbon and energy.

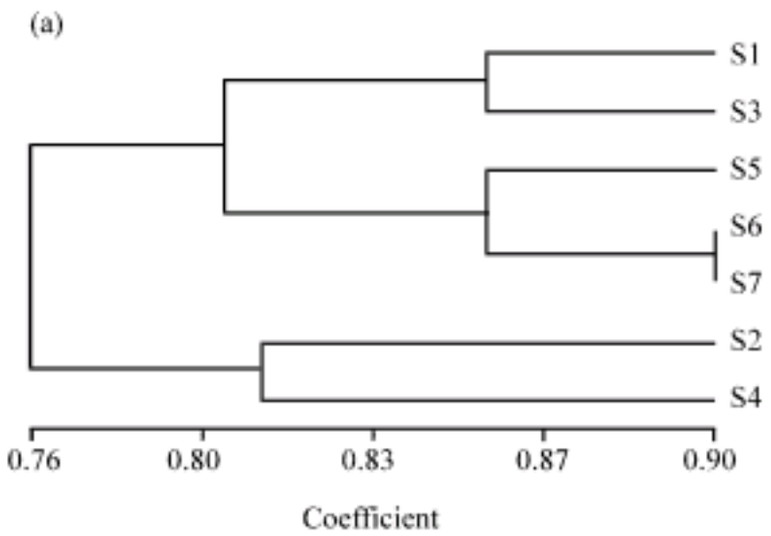

Fig. 2: Dendogram representing the bacterial diversity based in 16S banding pattern, using data presented in Table 2. A cluster analysis was performed with simple matching, as indicated in material and methods. (a) Seep location; (b) Control. Horizontal line indicates the correlation coefficient, limited from 0.7 to 1

Given that bands were counted as units and no band intensity was recorded, we could not investigate the ratios of different bacterial populations among the samples.

Taxonomic determination: 16S rDNA of *in vitro* propagated bacteria were cloned, sequenced and analyzed as described in material and methods. Eight independent, different rDNA sequences were obtained from these assays (Table 4). A significant homology of 98-99% was observed at DNA sequence level, were the genera *Marinobacter, Idiomarina, Marinobacterium* and *Frateuria*, as well as an unknown bacterium were present. The aforementioned genera have already been described to be present in marine habitats; however, their *in vitro* TPH-degrading capacity is a new finding, as described in the present study.

DISCUSSION

To evaluate the diversity of bacterial communities of shallow (≥100 m depth) oil seep marine sediments from the Southern Gulf of Mexico, a shallow spring of both oil and gas selected; contrasting to other seeps which have mostly gas or an asphaltic base (Broadhead *et al*., 1998). As indicated in results, the microbial diversity consisted to be similar when compared to control areas, however, the molecular identification of *Marinobacter, Idiomarina, Marinobacterium, Frauteria* and an unknown bacterium provided insights on the aim of the present study. The geochemical properties of seep sediments were characterized as well as their microbial diversity in oil seep and control sediments.

Although, the seep location is known to be a prominent and active oil generation and migration site, petroleum was unequally distributed in the different strata studied (Pellón de Miranda *et al*., 2004). A similar heterogeneous, patchy oil distribution in space and time was already seen in other shallow seep located in the Coal Point area, California (Allen *et al*., 1970; Broadhead *et al*., 1998). The discontinuous migration of petroleum through crevasses from subsurface deposits towards the surface creates zones with uneven oil concentration (Reed and Kaplan, 1977), depending on strata mechanical properties.

As established, bacteria have a restricted capacity to grow in laboratory conditions, likely due to the inability to simulate their habitat. Consistent with this fact, we could identify risotypes in seep samples where no colony forming units were observed in the tested media. The presence of uncultivable bacteria has been detected in some coastal and oceanic environments (Harayama *et al*.,

2004). As expected, petroleum hydrocarbons modulate both the diversity and the bacterial community structures in sediments. LaMontagne *et al*. (2004) reported that hydrocarbon seepage reduced the bacterial diversity determined by TRFLP in sediments of the coal oil point seep. However, present results showed that the bacterial diversity was slightly increased in the seep location; further, we observed the highest assortment of colony morphologies at a depth where a remarkable good generative potential of gas and oil was found. A biochemical approach, identifying *in situ* metabolic capacities would explain such differences.

Bacterial diversity should be understood in a theoretical frame that considers other parameters, such as temperature, O_2, nutrient availability and season of the year (Balser *et al*., 2001). Indeed, Broadhead *et al*. (1998) noticed a significant decrease in the bacteria population occurred during an exposure to a large amount of petroleum (such as in a sudden oil spill), followed by a stimulatory period and then by a gradual return to normal, once the TPH has been dispersed. The observed risotype variation did reflex the TPH content within the samples, as noted in the cfu counts for heterotrophic and TPH-degrading bacteria, a similar behaviour was observed in the Isla Vista seep sediments (California), rather than in the TPH-free sediments (Bauer *et al*., 1990). The presence of common bacterial risotypes found between locations suggests the presence of facultative bacteria, characteristic of microbial communities largely adapted to oil degradation (LaMontagne *et al*., 2004), with unique metabolic adaptations to this stringent environment (NAS, 2003). Despite the present study was focused on bacteria profile, it is important to mention the presence of yeast and mycelium-forming fungi in the microbial communities. *In situ*, all the microorganisms could have different ecological niches. Taken these facts together, the whole community could be playing an important role in the diagenesis of high and continuous carbon inputs, as well as in the establishment of some local food webs in marine oil seeps, as stated by Bauer *et al*. (1988). Since a limited number of microorganisms were identified by sequencing, it is assumed that the technique did not allow an exhaustive detection of the living bacteria, since no redundancy was observed. For this reason, it is possible that we also failed to detect Archaeae, largely expected in those environments. On the other hand, proteobacteria represented all the identified microorganisms, probably due to their phenotypic plasticity, including the degradation of petroleum hydrocarbons (Harayama *et al*., 2004). Finally, the identification of privative bacteria allows us to propose their identification as indicators of the presence of TPH in marine seeps. Likewise, it will be important to identify microorganisms in shallow and deep marine oil seeps.

ACKNOWLEDGMENTS

We thank Alejandro Ortega, Manager of Project D15-IMP for providing the sediment samples and geochemical analyses; Sylvie LeBorgne (IMP) and Gerardo Zúñiga (ENCB) for training in bioinformatics. MCR-H was an IMP fellow. This study was supported by CONACyT-SAGARPA 2004/27 to BXC, CONACyT 50769 to R. Ruiz-Medrano and D23-IMP to LFL.

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